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REVIEW ARTICLE

Enhancing the nutritional profile of rice by targeting starch branching enzymes using CRISPR/*Cas9*

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Abstract

Rice is a fundamental staple in many Asian countries; however, excessive consumption can lead to significant health concerns, including diabetes. One effective strategy to mitigate these concerns is to increase the amylose content in rice, which enhances its resistant starch (RS) levels. Higher RS not only improves the nutritional profile of rice but also positively impacts its cooking qualities, offering various health benefits. Recent research highlights the role of dietary fibers like RS in modulating gut microbiota composition, presenting a promising approach for addressing non-communicable diseases. RS enhances the fermentation activity of gut microbiota, leading to production of beneficial metabolites that support gut barrier function, exhibit anti-inflammatory properties and influence metabolic pathways related to obesity and diabetes. This multifaceted impact on chronic disease outcomes emphasizes the need for rice varieties with increased amylose and consequently higher RS levels, to meet consumer nutritional demands. CRISPR/*Cas9*, a powerful genome editing tool, allows precise modifications of the targeted genes. This technology can effectively edit starch synthesis-related genes in rice to enhance starch content. This review focuses on the application of CRISPR/*Cas9* in increasing RS content in rice and the potential health benefits it could provide to populations that rely on rice as a dietary staple. By integrating genetic innovation with nutritional science, healthier rice varieties can be developed, that align with the dietary needs of consumers.

Keywords

amylose; genome editing; resistant starch; rice; starch branching enzymes

Introduction

Rice (*Oryza sativa* L.) is a staple food for over 50% of the global population, particularly in Asian countries, serving as a critical source of nutrition and energy (1). Starch in rice, consisting of amylose and amylopectin, plays a key role in its nutritional and functional properties. Based on its digestion characteristics, starch is classified as rapidly digestible, slowly digestible and resistant starch (Table 1). Amylose, a linear polymer, is associated with resistant starch (RS), which resists digestion in the small intestine and undergoes fermentation in the colon, contributing to improved gut health and reduced glycemic response (2-4). High RS content in rice is linked to potential health benefits, including better glycemic control and reduced risks of metabolic disorders such as diabetes and cardiovascular diseases. The nutritional profile of rice is closely related to its amylose content (AC) and amylopectin structure

Table 1. Classification of starches, their occurrence and digestion characteristics (3, 4)

Type of starch	Rapidly digestible starch	Slowly digestible starch	Resistant starch
Occurrence	Freshly cooked starchy food	Most raw cereals	Foods rich in starch with more amylose
Digestion rate	Rapid digestion	Slow, but complete digestion	Resists digestion in the small intestine
Place of digestion	Stomach	Small intestine	Fermented in the colon by gut microbes
Time required for digestion	~ 20 minutes	20-120 min	>120 min

(5, 6). Varieties with high AC are preferred by consumers for their non-sticky texture after cooking and are characterized by a lower glycemic index (GI) (7). Consequently, rice varieties enriched in amylose and RS hold significant promise for addressing diet-related chronic diseases (8). As cereal crops rich in AC are not widely available, there is an increasing need to develop cereal crops high in AC and thus RS, to address the rapidly growing challenges in public health nutrition (9).

Both amylose and amylopectin are glucan polymers formed by glycosidic linkages between glucose monomers. Upon digestion these bonds are broken down by digestive enzymes and glucose is released. Amylose is made up of α-D-glucose units connected by α-1,4-glycosidic bonds, forming a helical structure. The biosynthesis of amylose occurs in the chloroplasts of plant cells and is mediated by the enzyme, granule bound starch synthase (GBSS), which catalyzes the addition of glucose units to the growing chain. Comparing with *japonica* cultivars, *indica* cultivars have significantly higher AC (10). Amylopectin is the branched polymer of glucose where the linear amylose chain is branched via α-1,6-glycosidic bonds. For biosynthesis of amylose and amylopectin, the combined activity of many enzymes and their isoforms is required, which are termed as starch synthesis-related genes (SSRGs) (Fig. 1). Manipulating these genes can enhance AC through methods such as over-expressing GBSS or suppressing starch branching enzymes (SBEs), starch synthases (SS) and starch de-branching enzymes (DBEs) (11-17). However, over-expression of GBSS results in only a limited increase in AC, likely due to the scarcity of reducing ends in amylose and competition for substrates with amylopectin (11). Additionally, the increase in AC observed in SS mutants is less pronounced compared to that in SBE-edited mutants (13, 18-20). Previous studies utilizing chemical mutagenesis or RNA interference (RNAi) have demonstrated that SBEs significantly influence the structure and physical properties of starch, leading to significantly higher RS levels in cereal crops (21-25). Therefore, targeting SBEs has emerged as the most common strategy for producing high AC in various crop species, including rice, barley, wheat, maize and other starch crops.

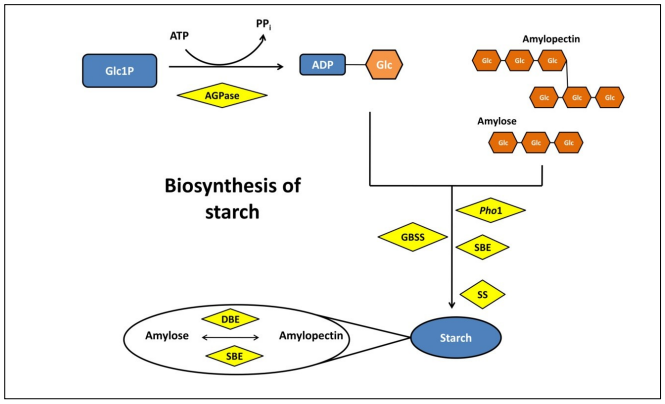

Fig. 1. Major enzymes involved in the biosynthesis of starch in plants.

(ADP-Adenosine diphosphate, AGPase - ADP-glucose pyrophosphorylase, ATP-Adenosine triphosphate, DBE- De-branching enzyme, GBSS- Granule bound starch synthase, Glc- Glucose, Glc1P-Glucose-1-Phosphate, *Pho**1***-Plastidial phosphorylase 1, PP_i- Pyrophosphate, SBE- Strach branching enzyme, SS- Starch synthase)

Due to the limitations in precision and efficiency of conventional methods, they have been surpassed by advanced genome editing tools in recent times. One such tool is CRISPR/*Cas9* (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9), originally derived from the bacterial defense mechanism against viruses. In this system, a single guide RNA guides a nuclease to the target site, which causes double-stranded breaks. These breaks will be repaired by the cellular machinery resulting in mutations. It is employed to induce site-specific mutations for genome editing purposes, whose specificity and efficiency have made it the tool of choice in this field (26). The mutations will be mostly small insertions, deletions, substitutions or large fragment substitutions. Repair through non-homologous end joining (NHEJ), an error prone mechanism, will result in insertion/deletions (indels). On the other hand, repair through homology directed recombination (HDR) pathway, which utilizes flanking sequences or an external repair template, will result in large fragment substitutions. The utilization of CRISPR in crop improvement includes selection of target site(s), designing of guide RNA(s), cloning into suitable vector for plant transformation, selection of proper methods for vector delivery into the plants and generation and screening of the mutants (27). CRISPR/*Cas9* system in plants is capable of targeting one or multiple genes simultaneously. Since the first application of this system in 2013 for plant genome editing, it has been utilized in many crop species for yield and quality improvement. Advancements in CRISPR systems such as CRISPR-Cf1, base editing, prime editing CRISPR-inducible genome editing and epigenome editing have paved way for production of high-quality crop species.

It has also been utilized in many crops to manipulate the starch synthesis pathway to increase the RS content and it could also be applied to the development of rice varieties with elevated RS levels. This review focuses on the application of CRISPR/*Cas9* technology in targeting starch branching enzymes to enhance the RS content in rice. By improving the nutritional attributes of rice, this strategy not only aligns with consumer preferences but also would address global health challenges related to metabolic disorders.

Resistant starch (RS)

RS is prevalent in a variety of foods, such as grains, cereals, legumes, seeds, vegetables and certain nuts. It is categorized into distinct types namely, resistant starch type 1 (RS1), resistant starch type 2 (RS2), resistant starch type 3 (RS3),

resistant starch type 4 (RS4) and resistant starch type 5 (RS5) based on the mechanisms through which it resists digestion by host enzymes (4).

RS is characterized by high AC and distinct amylopectin structures. The potential of starch to evade digestion in the small intestine is influenced by multiple factors with surface microstructure of starch being a significant one. Relative AC, density of amylopectin branch chains and crystallinity are believed to influence the texture and porosity of the starch granule surface. The amylopectin side chains and the amylose chains organize themselves into helical conformation and form crystals of two types *viz.* type A and type B (28, 29). Starch granules exhibiting a crystalline surface demonstrate greater resistance to enzymatic hydrolysis when compared to granules with an amorphous surface. In type A crystals, enzymatic hydrolysis occurs extensively, whereas in type B crystals, hydrolysis is limited to the surface of the crystal (4). Surface crystallinity and intermolecular networks of starch are altered by retrogradation and cross-linking, leading to increased resistance to hydrolysis (30).

Relationship between high amylose and RS

Linear amylose and branched amylopectin make up starch, among which amylose particularly has a significant impact on how starch functions. Research shows that starches with high AC are generally more resistant to digestion than those with low AC. This increased resistance is due to structural differences, as amylose has fewer branches compared to amylopectin, which makes it harder for enzymes to break it down (31). High-amylose starches (those found in high-amylose wheat and rice) typically have more proteins bound to their granular surfaces. These proteins can create a barrier that reduces enzyme binding to the starch and results in reduced digestion rate (31, 32). Foods high in amylose are linked to lower blood glucose levels and a slower rate of stomach emptying compared to foods with lower AC (33). Rats fed with wheat grains with an elevated AC (>70%) had better colonic functional indicators, such as concentration of short-chain fatty acids (SCFAs), than rats given ordinary wheat grain (22). This shows that food with high AC has a significant potential to benefit health by supplying RS. The SCFAs produced by fermentation of RS in the human gut confers immense health benefits (Fig. 2). The use of genomic techniques to create starch with high AC and enhanced amount of RS is therefore the subject of extensive investigation.

Enzymes involved in starch synthesis

Starch biosynthesis in plants occur due to the combined action of several enzymes such as ADP-glucose pyrophosphorylase (AGPase), starch synthase (SS), branching enzyme (BE), debranching enzyme (DBE) and plastidial starch phosphorylase (*Pho1*).

The enzyme AGPase catalyzes the formation of ADP-glucose from glucose-1-phosphate and ATP. This reaction is a key regulatory step in the biosynthetic pathway. SS is responsible for the elongation of the amylose chain by adding glucose units from ADP-glucose, forming α -1,4-glycosidic bonds. Though it is linear, minor branching also occurs in amylose due to the action of branching enzymes. Isoforms of SS include granule bound starch synthase (GBSS) which is responsible for amylose formation and soluble starch synthase (SSS) which is responsible for amylopectin synthesis along with branching enzymes and debranching enzymes. The three isoforms of SS, namely SSI, SSIIa and SSIII, are found in the amyloplast stroma and are basically involved in the amylopectin biosynthesis by the elongation of pre-formed α-glucans of varying length (which are produced from the actions of varying

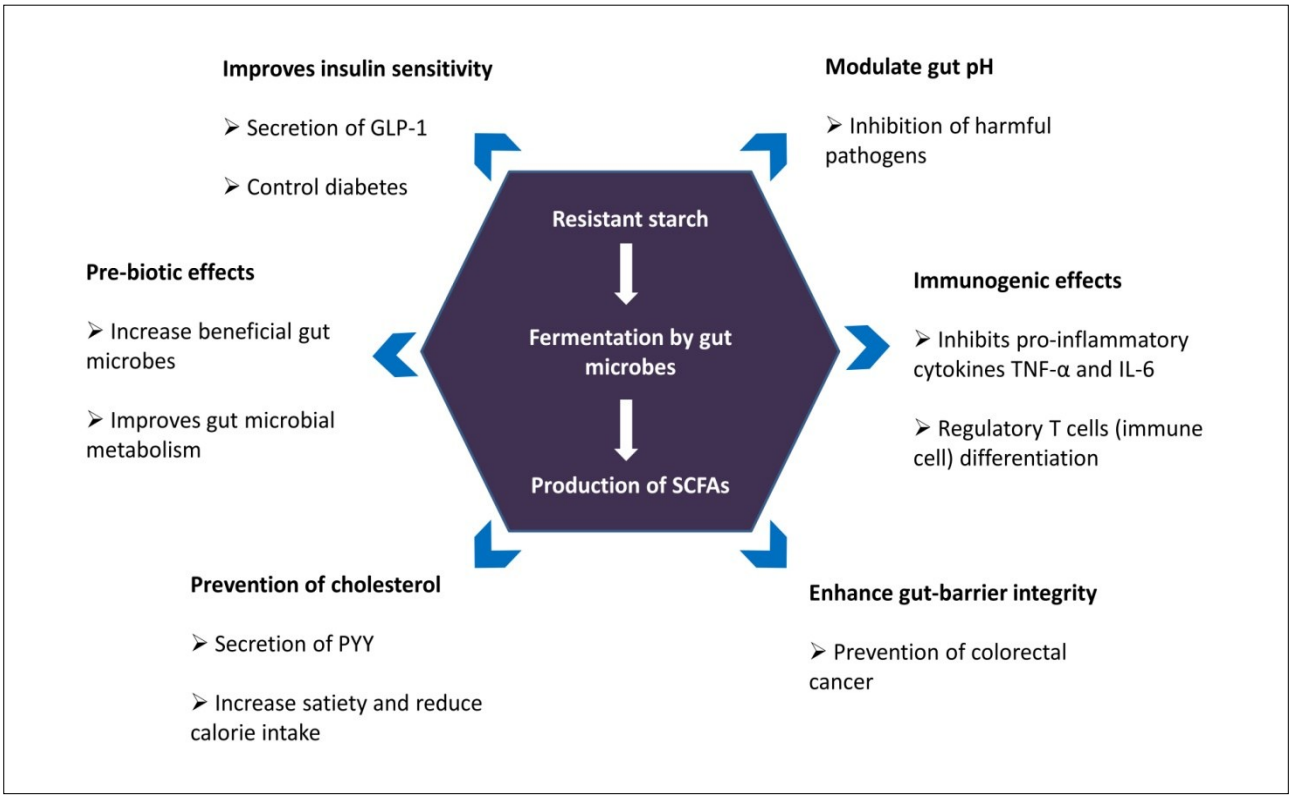

Fig. 2. Potential health benefits of resistant starch consumption.

(GLP-1 - Glucagon-like peptide, IL-6 - Interleukin 6, PYY - Peptide tyrosine tyrosine, TNF-α - Tumor necrosis factor-alpha)

enzymes) (34).

The key enzyme responsible for the branching of amylopectin is the SBE, which introduces α-1,6-glycosidic linkages by transferring a segment of the glucan chain to a different position on the same or another chain. This enzyme is critical for the formation of amylopectin (35). SBEs catalyse a non-reversible reaction where the transglycosylation of α-1,4-glycosidic linkages results in the formation of α-1,6-branch points within α-1,4-glucans (34).

DBEs hydrolyse α-1,6-glycosidic linkages of polyglucans. These are divided into two types based on substrate specificity- isoamylases (debranches glycogen, phytoglycogen and amylopectin) and pullulanase (attacks pullulan and amylopectin) (35). The action of DBEs is essential for the removal of irregular amylopectin chains to ensure an ordered branching. The role of *Pho1* enzyme in starch synthesis, however, is unclear (35, 36).

Starch branching enzymes in rice

SBEs belong to the glycoside hydrolase 13 (GH13) family of enzymes within the Carbohydrate-Active Enzymes (CAZy) database (37). These enzymes play a crucial role in starch biosynthesis by catalyzing the cleavage of α-1,4 glycosidic bonds in amylose and facilitating the formation of α-1,6 glycosidic linkages in amylopectin. Isoforms of SBEs occur in rice, each contributing uniquely to the branching density and structural composition of amylopectin. While three primary isoforms SBEI, SBEIIa and SBEIIb are widely reported, a fourth isoform, SBEIII, has been described by some studies (9, 38, 39).

SBEI plays a significant role in the biosynthesis of amylopectin, particularly in the formation of intermediate chain types such as B_1, B_2 and B_3 chains (35, 40, 41). A-chains are connected to other chains via glucose units at their reducing ends, while C-chains contain free reducing ends. B_1-chains are located within a cluster, whereas B_2- and B_3-chains interconnect multiple clusters (subscript numbers indicate the number of clusters linked) (35). Research suggest SBEI is also involved in the synthesis of long amylopectin chains, although its role in amylose synthesis is limited to the elongation of short chains, with little or no contribution to longer chains (42). In a study with kinetic properties of SBEI, it exhibits a lower Km value for amylose compared to SBEIIa, signifying a stronger affinity for linear glucans. SBEI is likely responsible for synthesizing both intermediate and long amylopectin chain types (43).

The SBEII isoforms, SBEIIa and SBEIIb, are encoded by the genes *OsSBEIIa* and *OsSBEIIb*, respectively. They share approximately 80% sequence similarity but exhibit distinct expression profiles due to evolutionary sub-functionalization (44). SBEIIa is predominantly expressed in leaves and non-storage tissues, while SBEIIb is primarily expressed in the endosperm of seeds, where it plays a critical role in starch biosynthesis. SBEIIa preferentially transfers short amylopectin -type chains. SBEIIb transfers even shorter chains than SBEIIa, forming A- and B_1- chains in storage tissues. These short chains are subsequently extended by SS enzymes to form the final structure of amylopectin. Mutations in SBEIIb result in the amylose extender (ae) phenotype, characterized by fewer branches, longer amylopectin chains and increased AC (41, 45). However, inactivation of SBEIIa or SBEI does not lead to significant morphological changes in seeds.

In vitro kinetic study demonstrates distinct preferences among the SBE isoforms for substrate chain length. SBEI exhibits broad activity, transferring a wide range of chains (degree of polymerization; DP ≤ 40), including both outer and inner chains of amylose and amylopectin (46). In contrast, SBEIIa transfers short chains (DP 6-15), while SBEIIb prefers shorter chains (DP 6-7). Notably, SBEIIa and SBEIIb lack the ability to attack inner chains, differentiating their activity from that of SBEI (40).

These isoforms can partially support or compensate for each other under specific circumstances. SBEIIa contributes to forming intermediate amylopectin chains in the absence of SBEI or SBEIIb but it cannot fully compensate for the combined absence of both (43). However, SBEI and SBEIIa alone or in combination, cannot complement the role of SBEIIb in the formation of A type chains.

The fourth isoform, SBEIII, is implicated in forming α-1,6 linkages, although its role remains less extensively characterized (9, 39). In a study with knockout mutants of different combinations of SBEs in rice, the SBEIIa mutant had no significant changes in the proportion of amylose chains or intermediate and long amylopectin chains. In the SBEIIb mutant, the crystallinity of the amylopectin chains changed from A type to B type and the proportion of long and intermediate chains increased. SBEI deficiency increased the proportion of short amylopectin chains and decreased long and intermediate chains (47). These isoforms collectively contribute to the structural complexity of amylopectin in the order SBEIIb > SBEI > SBEIIa, ensuring the balance of chain length and branching density that are critical for starch functionality.

The CRISPR/ Cas9 system

Genome editing technology is an efficient way to make modifications in an organism's genomic DNA. The core of genome editing is the use of sequence-specific nucleases (SSNs) that creates double stranded breaks (DSBs) in the DNA. These breaks are generally repaired by two important pathways, NHEJ and HDR. At present, there are four major SSNs such as meganucleases or homing endonucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspersed short palindromic repeats - CRISPR associated protein 9 (CRISPR/ *Cas9*) (48). Each of these technologies offers unique mechanisms for targeting and altering genetic material, contributing to advancements in fields such as agriculture, medicine and genetic research. CRISPR/*Cas9* is an efficient genome editing tool that is faster, cheaper and precise even at multiplexing level (49). Hence, it is widely adopted compared to other systems of genome editing.

CRISPR/*Cas9* is a system developed from the bacterial adaptive immune system for resistance against virus. In bacteria, these systems store the invading viral DNA fragments in repetitive spacer arrays, which upon further processing will yield the CRISPR RNA (crRNA). When the same virus attacks the bacterium again, these crRNAs act as guide and direct the molecular machinery containing *Cas9* proteins to cleave the invading viral DNA. Research revealed that the combined

action of crRNA and another trans-activating crRNA (tracrRNA) is essential to guide the *Cas9* protein to the target (26, 50, 51).

Later, this was developed into the CRISPR/*Cas9* genome editing system, which can edit the genomic regions by causing DSBs in the DNA (52). The major components of CRISPR/*Cas9* include the *Cas9* protein and the single guide RNA (sgRNA) (Fig. 3). *Cas9* protein is a RNA-dependent DNA endonuclease. *Cas9* protein from *Streptococcus pyogenes* (*S. pyogenes*) was first utilized for genome editing and is widely used due to its specificity and high activity. The crRNA and tracrRNA are combined into sgRNA (53, 54).

To direct the CRISPR/*Cas9* complex to the region of interest, 20 nucleotides (nts) at the end of crRNA can be modified so that they are complementary to the target region of the genome; the 20 nts sequence is termed as guide RNA (gRNA). *Cas9* nuclease has two lobes, nuclease lobe (NUC) and recognition lobe (REC). REC recognizes and binds with the tracrRNA (50). NUC lobe is again divided into three - Protospacer adjacent motif (PAM) interacting domain, HNH domain and RuvC domain. PAM is located immediately downstream of the target site in the non-target strand and is necessary for binding of the *Cas9*. PAM region carries the sequence, 5'-NGG-3', in case of *Cas9* protein from *S. pyogenes* (55). *Cas9* binds to PAM region through the PAM interacting domain and this recognition causes local unwinding of the DNA at target site so that the gRNA binding can occur. The binding of gRNA causes conformational changes in the *Cas9* and the NUC domain gets activated (54, 56). Being an endonuclease, *Cas9* creates DSBs at the target site in the genome, three nucleotides upstream of the PAM region. The target strand and the non-target strand are cleaved by the HNH domain and RuvC domain of the *Cas9*, respectively. These double stranded breaks, which upon being repaired by the repair machinery of the cell, either through NHEJ or HDR, results in site specific mutations (57).

Cas9 can be converted into RNA-guided nickases by disabling either of its NUC domains through alanine substitutions in the catalytic regions. Specifically, the D10A substitution deactivates the RuvC domain, while the H840A substitution deactivates the HNH domain. These modified nickases are capable of introducing single-stranded breaks or nicks in either the target strand or the non-target strand of DNA. When both the HNH and RuvC domains are inactivated, *Cas9* becomes dead *Cas9* (d*Cas9*), functioning as an RNA-guided DNA-binding protein without nuclease activity (58, 59). Currently, the plant CRISPR/*Cas9* system and its derivatives exhibit a wide range of genome-editing capabilities such as gene knockdown, knock-in and knockout, including expression activation. Moreover, it also has the ability to edit multiple genes simultaneously (multiplex genome editing).

CRISPR/Cas9 based genome editing in rice

Since its discovery, the CRISPR/*Cas9* system has been successfully applied in model plant system, *Arabidopsis*, and also in many crop plants, among which rice is the most extensively targeted one (60). The reason could be due to the small genome size of rice, availability and access to more sequence data and genetic resources and ease of transformability. Utilization of CRISPR/*Cas9* system in rice has been demonstrated by several workers (Table 2). Though many methods such as *Agrobacterium tumefaciens,* biolistic gene gun, protoplast, floral dip and microinjection are available for the delivery of vector containing CRISPR/*Cas9* construct into the plant, *Agrobacterium* mediated transformation is widely preferred (61-66). It has been applied to enhance key traits in rice such as yield related traits, flowering time/heading date, stress tolerance, nutrient efficiency and quality traits.

Yield improvement

CRISPR/*Cas9* technology is utilized to improve plant architecture and grain related traits. Several genes and quantitative trait loci (QTLs) involved in these traits have been

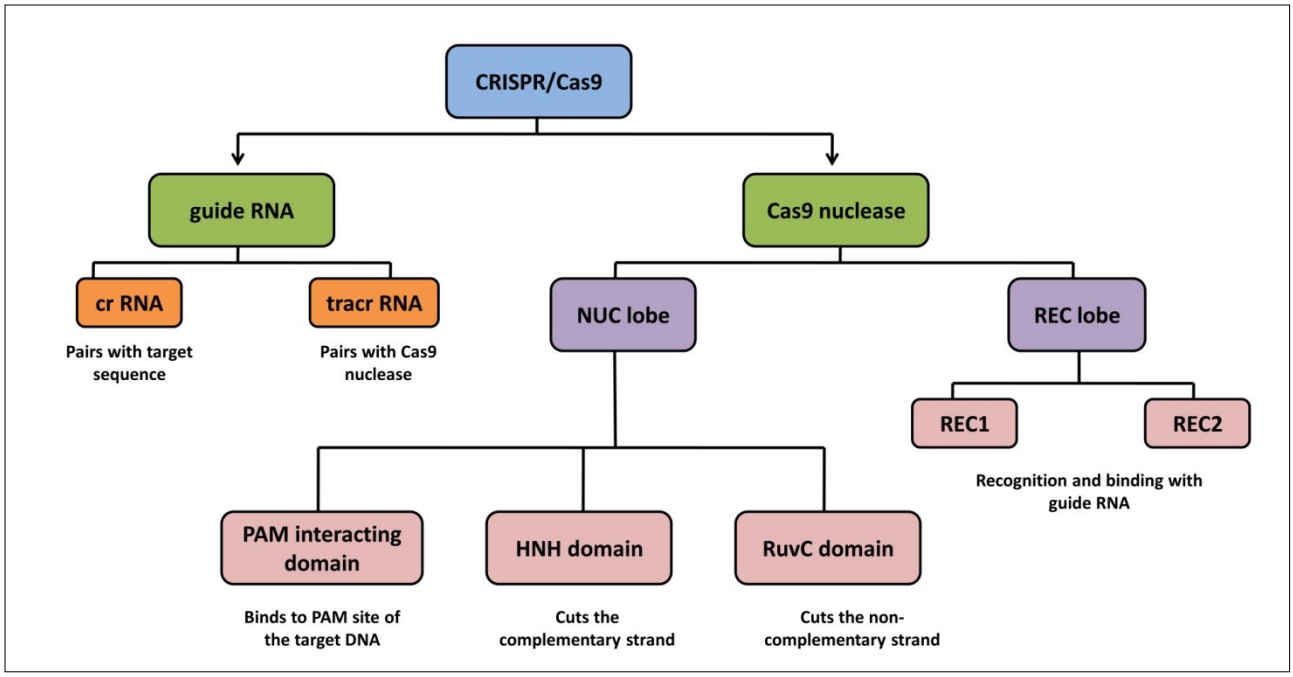

Fig. 3. Components of the CRISPR/*Cas9* system and their functions.

(CRISPR - Clustered regularly interspaced short palindromic repeats; *Cas9*- CRISPR associated protein 9; crRNA- CRISPR RNA; tracr RNA - trans-acting CRISPR RNA, REC- Recognition; NUC- Nuclease; PAM- Protospacer adjacent motif).

Table 2. CRISPR/*Cas9* studies on genes controlling agronomically important traits in rice

Trait	Targeted gene (s)	Strategy	Improvement	Reference
Abiotic stress tolerance	*OsEPSPS*	Intron targeting	Glyphosate resistance	(79)
	ALS	Multiple discrete point mutations in ALS gene	Chlorosulfuron resistance	(80)
	OsSAPK2	Targeting mediators of ABA signaling pathway	Drought tolerance	(76)
	TIFY1a, TIFY1b	Exons of both genes targeted	Cold tolerance	(78)
	OsCS511	Increasing synthesis of various ROS-related proteins	Cold tolerance	(77)
Aroma	*BADH2*	Increasing 2-acetyl-1-pyrroline content	Enhanced fragrance	(83, 84)
Biotic stress tolerance	*OsERF922*	Multiple mutations within the transcription factor	Enhanced resistance to blast disease	(72)
	OsERF65	Transcription factors in ROS homeostasis	Sheath blight resistance	(73)
	OsSLR1	Gibberellic acid (GA) signaling pathway	Sheath blight and lodging resistance	(123)
	OsMESL	ROS accumulation mediated broad spectrum disease resistance	Resistance to rice sheath blight, bacterial blight and blast	(74)
	Bsr-d1, *Pi21* and *ERF922*	Up regulation of SA- and JA-pathway associated genes	Resistance to rice blast and bacterial blight	(124)
	eIF4G	YVV residues of translation initiation factor 4	Resistance to *Rice tungro spherical virus*	(75)
Male sterility	*CSA*	Mutations in thermosensitive male sterility genes	Photoperiod controlled male sterile lines	(82)
	TMS5	Mutations in thermosensitive male sterility genes	Development of TGMS lines	(81)
Nutritional improvement	*OsNRAMP5*	Knockout of metal transporters	Low cadmium content	(85)
	OsGluA2,*OsAAP6, OsAAP10*	Knocking out positive regulators of grain protein content	Reduced grain protein content and improved eating & cooking quality	(87, 88)
	GR2 (Golden rice 2) cassette	Targeted insertion of carotenoid biosynthesis cassette at genomic safe harbors in rice	Enhanced carotenoid content	(125)
	OsGAD3	Deletion of calmodulin binding domain	Increased Gamma-aminobutyric acid content	(89)
	OsVIT2	Mutations in vacuolar Iron Transporters	Increased Fe content in grains	(86)
	OsASTOL1	Ser189Asn point mutation	Selenium accumulation and arsenic tolerance	(90)
Starch biosynthesis	*GBSSI*	Reduction in GBSS activity in seeds	Reduced amylose content	(126)
	Waxy (*Wx*)	Creating mutant alleles of *wx* gene	Generation of Glutinous rice varities	(127)
	OsSBE	Mutation of starch branching enzymes	Generation of high amylose rice	(96)
Stomatal density	*OsEPFL9*	Early developmental genes	Regulates leaf stomatal density	(128)
Yield and quality improvement	*CCD7*	Strigolactone biosynthesis pathway	Increased tiller number	(68)
	Ehd1	Multiple mutations in the promoter	Improved yield traits	(69)
	Gn1a, DEP1, GS3 and *IPA1*	Improvement of grain number, panicle architecture, grain size and plant architecture	Enhanced yield	(67)
	GW2, GW5 and *TGW6*	Targeting negative regulators of grain weight	Improvement of grain weight	(129)
	Hd2, Hd4 and *Hd5*	Targeting genes negatively affecting heading date	Early maturity of rice varieties	(130)
	OsCPK18/OsCPK4	Phosphorylation pathways	Improved yield and disease resistance	(70)
	PYLs	Regulatory components of the ABA receptor family of proteins	Improved growth and productivity	(71)

targeted for modification. Notably, editing of genes such as Gn1a, DEP1, GS3 and IPA1 has improved grain number, panicle architecture, grain size and plant stature, all of which contribute to higher rice productivity (67). The disruption of CCD7, responsible for strigolactone biosynthesis, has led to increased tillering and altered plant height. This alteration enhances the number of panicles, setting the foundation for potential yield improvement (68). *Ehd1*, a gene regulating heading time, was modified by editing the promoter region to downregulate its expression. It resulted in delayed heading and improved agronomic traits, which could potentially expand planting areas and improve yields (69). In addition, multiplex genome editing with CRISPR/*Cas9* has been employed to target multiple QTLs associated with grain weight, facilitating rapid improvement in rice yield. *OsCPK18* and its paralog *OsCPK4*, regulate both growth and immunity. CRISPR/*Cas9* was used to edit the phosphorylation sites in *OsCPK18* and *OsMPK5*, enhancing their activity, which enhanced stress resilience alongside improved yield (70). Finally, modifying the PYL genes (PYL1-6, PYL 12), which are involved in abscisic acid signaling, has led to better growth and productivity under stress conditions, further boosting

yield potential (71).

Stress resistance

CRISPR/*Cas9* has proven to be a valuable tool in enhancing the resistance of rice to both biotic and abiotic stresses. In terms of biotic stress, CRISPR/*Cas9* has been used to target genes such as *OsERF922*, *OsERF65*, *CIPK31* and *OsMESL*, leading to mutations that improve resistance to diseases like rice blast, bacterial blight and sheath blight disease, without introducing foreign transgenes (72-75). Different editing strategies, such as sgRNA-based targeting and multiple sgRNAs, were employed to generate specific mutations. These mutants showed reduced disease severity and maintained agronomic traits. Additionally, CRISPR/*Cas9* was used to edit the *eIF4G* gene in the rice tungro spherical virus (RSTV)-susceptible variety, IR64, conferring RSTV resistance and enhanced yield (75).

In addressing abiotic stress, CRISPR/*Cas9* has enabled the precise editing of key genes like *SAPK2*, *TIFY1a*, *TIFY1b* and *OsCS511* to enhance rice's tolerance to heat, drought, salinity and cold (76–78). Strategies such as gene knockout, frameshift mutations and targeted insertions were applied to modify these genes, improving stress resilience. For example, the disruption of *SAPK2* resulted in increased stress sensitivity, while upregulation in wild-type plants led to enhanced tolerance. CRISPR/*Cas9* has also been utilized to confer herbicide resistance by editing the *EPSPS* and *ALS* genes. In the case of *EPSPS*, mutation frequencies of 2.0% and 2.2% were achieved, while the *ALS* gene was edited with dual-guide RNAs and DNA repair templates to generate homozygous herbicide-resistant plants in a single generation (79, 80). These findings underscore the significance of CRISPR/*Cas9* in improving both biotic and abiotic stress resistance, demonstrating its potential as a powerful tool for crop improvement.

Male sterility

Hybrid rice breeding plays a critical role in enhancing rice production, where the use of male sterile lines is a fundamental strategy for successful cross-breeding. Traditionally, male sterility has been regulated by environmental factors such as temperature (thermo-sensitive genic male sterility; TGMS) or day length (photoperiod-sensitive genic male sterility; PGMS). However, with the advent of CRISPR/*Cas9* technology, precise genome editing can be used to produce transgene-free sterile lines in rice. CRISPR/*Cas9* was used to induce specific mutations in the most widely used TGMS gene-*TMS5*. Using the *TMS5ab* construct, the researchers generated 11 new TGMS lines within a year, demonstrating that CRISPR/*Cas9* technology can significantly expedite the breeding of sterile lines, thereby facilitating the exploitation of heterosis (81). CRISPR/*Cas9* was also utilized to target the *CSA* gene in *japonica* rice varieties, resulting in the development of reversible photoperiod-sensitive genetic male sterile (rPGMS) lines. These lines exhibit male sterility under short-day conditions and partial fertility under long-day conditions, making them highly valuable for hybrid rice breeding (82).

Aroma

The *OsBADH2* gene, which encodes the enzyme betaine aldehyde dehydrogenase, is crucial for controlling the aroma in rice grains. Mutations in this gene lead to the production of 2 -acetyl-1-pyrroline, the compound responsible for the characteristic fragrance of aromatic rice varieties. The *Badh2* gene was edited using CRISPR/*Cas9*, resulting in mutants with increased 2-acetyl-1-pyrroline content and improved aroma, providing a foundation for fragrant rice breeding (83, 84).

Nutrient enrichment

As consumer demand increasingly shifts toward healthier and more nutritionally enriched food products, there has been a growing emphasis on developing new food items to meet these preferences. In this context, genome editing using CRISPR/*Cas9* has become a highly effective tool for enhancing crop quality by precisely targeting genes that regulate nutrient composition.

OsNRAMP5, a gene involved in iron uptake and *OsVIT2*, the gene responsible for vacuolar iron transport were targeted to enhance iron bioavailability in rice. Mutation in these genes resulted in increased iron accumulation in rice grains and altered iron distribution within the plant, particularly increasing iron levels in the rice grain without affecting agronomic performance (85, 86). Similarly, CRISPR/*Cas9* was used to target *OsAAP6* and *OsAAP10* genes that regulate amino acid transport and protein content in rice. The knockout of these genes led to a significant reduction in grain protein content (GPC), which in turn improved the cooking and eating quality of rice by lowering AC and enhancing the texture (87, 88). *OsGAD3*, a gene encoding glutamate decarboxylase, was modified to boost the levels of gamma-aminobutyric acid (GABA) in rice seeds. Researchers used the CRISPR/*Cas9* genome editing system to remove the Ca^{2+}/calmodulin binding domain (CaMBD), an autoinhibitory domain, from *OsGAD3* which resulted in a seven-fold increase in GABA content, as well as improved seed weight and protein content (89). Finally, the *astol1* mutant, identified through CRISPR/*Cas9*, displayed enhanced sulfur and selenium assimilation, leading to improved arsenic tolerance and reduced arsenic accumulation in rice grains. The *astol1* mutation involves a gain-of-function alteration rather than a typical CRISPR/*Cas9* knockout or knock-in approach. The mutation leads to the activation of the serine-acetyltransferase enzyme, which plays a critical role in enhancing sulfur and selenium uptake (90). These modifications were achieved without significant yield penalties, demonstrating that CRISPR/*Cas9*-mediated genome editing offers a precise and efficient method for improving multiple aspects of rice quality, from nutrient enrichment to stress resilience.

From the above studies, it becomes evident that the CRISR/*Cas9* has been efficiently utilized in rice. By using sgRNA constructs with different vectors and promoters, increased efficiency of gene knockout was achieved that led to the development of mutant populations with high mutation frequencies, higher variability and accuracy (91, 92). CRISPR-*Cas9* system was also employed in multiplex genome editing (MGE) approaches which use multiple sgRNAs to modify the rice genome. The efficacy of this method is further evaluated through the expression of multiple sgRNAs under U3/U6 promoters (93). As the CRISPR/*Cas9* system is well established in rice, it could be used as a potential tool to manipulate genes

of the starch biosynthesis pathway, to increase the rice RS content.

Genome editing to enhance starch content in rice

As discussed earlier in the introduction, the most common strategy for generating high AC in different species, such as barley, wheat, maize and other starch crops, is through suppression of SBEs. Inhibiting these enzymes in cereal endosperm through CRISPR/*Cas9* mediated genome editing would decrease the branching degree, leading to a significant increase in the amount of AC and RS in rice grains (94). This hypothesis is supported by several works on SBEs, where the reduction of its activity led to decreased branching in the amylopectin and increased AC (13, 22, 95-99). In rice, downregulation of the *OsSBEIIb* gene is achieved by means of chemical treatment or radiation through hairpin RNA (hp-RNA) mediated RNAi or by targeted mutation through CRISPR/*Cas9* (45, 100); that has resulted in increased AC. In addition to rice, significant changes in AC were observed through downregulation or elimination of SBEs using CRISPR/*Cas9* in different crop species (Table 3).

The downregulation of SBEs either by targeting a single SBE or a combination of SBEs has resulted in significant variation in the AC of rice (21, 99, 101). High amylose mutants were developed in rice through mutation of the SBEIIb genes and are known as the amylose-extender mutants (*ae*). In a study involving the *japonica* rice cultivar Nipponbare, mutations induced by CRISPR/*Cas9* in the *OsSBEIIb* gene resulted in the production of a non-functional protein that lacked catalytic activity. The homozygous mutants showed an increase in AC of up to 27%, which is 1.4-fold higher than that of the wild type and the RS content reached 17.2%. Targeting the *sbeIIb* locus in an elite low-glutelin *japonica* rice cultivar resulted in 1.8-fold increase in AC and increased RS content of 6% (102). Another study focused on the *japonica* cultivar *Kitaake*, targeting the *OsSBEI* and *OsSBEIIb* genes using CRISPR/*Cas9*. The *OsSBEI* mutants did not show any significant differences compared to the wild type. In contrast, the *OsSBEIIb* mutants exhibited an increase in AC of 25% and RS levels of 9% (96). This could be due to difference in expression pattern of these two genes, with SBEIIb being expressed in the rice endosperm produces more pronounced effects when disrupted. Another reason could be the chain length preference of the SBEs. Among the three major SBEs in rice (SBEI, SBEIIa and SBEIIb), SBEI transfers long and intermediary chains while SBEIIa and SBEIIb transfers short amylopectin chains. SBEI creates branch points with less frequency so that in a long amylopectin chain, the distance between two branches is quite long when compared to the branches created by SBEIIb. These long amylopectin chains have characteristics similar to that of an amylose chain, hence downregulation of SBEI produce no significant variation in the grain AC content. Targeting all four starch branching enzymes, SBEI, SBEIIa, SBEIIb and SBEIII, using multiplex CRISPR/*Cas9* genome editing was reported in the U.S. rice cultivar *Presidio*. Endogenous tRNA processing system was utilized for processing the gRNAs targeting the four SBEs. Various combinations of mutations in the SBE genes were reported, with mutants harboring alterations in all four SBE genes exhibiting a significant increase in AC compared to the wild type and other mutant lines. Additionally, an increase in RS content of up to 15% was observed (9). This study once again highlights the importance of SBEs in determining the AC of rice.

In addition to targeting SBEs, other genes involved in amylopectin biosynthesis, such as SS isoforms, can also be targeted to enhance AC. Combining mutations in both types of genes leads to a more significant increase in AC compared to individual mutations. Mutations in SSIIIa in rice results in a phenotype with 30.7% AC, whereas combining this mutation with SBEIIb mutants, the AC increases further to 45% in the ss3a/sbe2b double mutant (103). In summary, CRISPR/*Cas9* can be effectively used to increase the AC in rice varieties. These amylose-enhanced varieties would contain more RS, which offers health benefits to humans. While other genes can also be targeted to boost amylose levels, focusing on SBE genes is more advantageous, as the increase in AC in SBE mutants is considerably greater than in mutants of other genes.

Role of RS in glycemic control and metabolic health

Foods with high GI, such as processed carbohydrates and sugars, are rapidly digested and absorbed, resulting in a rapid increase in blood sugar levels. In contrast, low GI foods rich in protein, fiber and fat are digested and absorbed at a slower rate (104). Similar to a low-glycemic index diet, RS has the potential to lower postprandial glucose levels and may reduce the risk of metabolic syndrome, obesity and hypertriglyceridemia. RS3 consumption has proved to significantly reduce the mean blood glucose levels and total blood glucose in patients with type 2 diabetes (105, 106). The physical and chemical characteristics of various RS types vary, as does their reaction to a given host. In a study with mice, it has been demonstrated that RS

Table 3. Amylose level changes in different crop species by downregulation of SBEs

S.No.	Crop	Gene(s) targeted	Changes in amylose content (AC)	Reference
1.	Potato	*SBEII*	Increase upto 35%	(118)
2.	Potato	*SBEI* and *SBEII*	Increase upto 70%	(119)
3.	*Brassica napus*	All starch branching enzymes	Lower SBE enzyme activity and altered pattern of amylopectin chain length distribution	(120)
4.	Barley	*SBEIIa* and *SBEIIb*	Increase upto 70%	(97)
5.	Barley	All starch branching enzymes	Grains with almost entirely amylose	(13)
6.	Maize	*SBEIIb*	Increase upto 50-80%	(121)
7.	Maize	*SBEI* and *SBEIIb*	Increase >50%	(122)
8.	Wheat	*SBEIIa*	>70% increase	(22)
9.	Rice (*japonica*)	*SBEIIb*	Increase upto 25-30%	(98, 99)
10.	Rice	*SBEIIb*	Increase upto 25%	(100)

consumption increased beneficial microbial population and SCFA levels, resulting in reduction of high fat diet (HFD)-induced obesity (107).

The SCFAs produced by the gut microbes are essential for maintaining gut health through regulation of the luminal pH, mucus production, providing fuel for epithelial cells and effects on mucosal immune function. Lowering of gut pH by the SCFAs creates an unfavorable environment for the microorganisms that are pathogenic to the host. They also modulate host metabolic health through tissue-specific mechanisms related to glucose homeostasis and immunomodulation (5). Thus increasing gut-derived SCFA production could be a valuable strategy for preventing a wide range of health conditions and diet-related diseases in humans (108). Butyrate in particular is essential for the functioning of the colonocytes (the epithelial cells of the gut), aiding in their growth and repair. It also enhances the integrity of the gut barrier by regulating the proteins that forms the tight junctions between epithelial cells, thus preventing the leaky gut syndrome. This prevents infections and inflammations by inhibiting the growth of pathogenic bacteria in the gut. Butyrate has been known to reduce the production of pro-inflammatory cytokines and modulate immune responses, which can be beneficial in managing inflammatory bowel disease (IBD) and other chronic inflammatory conditions (109). Propionate has been linked to improved insulin sensitivity and helps control blood glucose levels, making it particularly relevant for individuals with type-2 diabetes. SCFAs can stimulate the secretion of hormones like glucagon-like peptide-1 (GLP-1) which plays a role in appetite regulation and glucose homeostasis (110, 111). Research suggests that SCFAs, particularly butyrate, may have protective effects against colorectal cancer. They can inhibit the proliferation of cancer cells and induce apoptosis in malignant cells. SCFAs may help lower cholesterol levels and reduce the risk of cardiovascular diseases by modulating lipid metabolism. Furthermore, SCFAs can influence the gut-brain axis, potentially impacting neuroinflammation and mental health (112). Thus, production of SCFAs by gut microbiota is a vital process that supports various aspects of human health and this process is fueled by RS consumption.

Conclusion

Gene editing technologies, especially the CRISPR/*Cas9* system, have become more significant in modern plant research. It is of immense advantage in developing varieties with improved traits including increased RS in staple food crops, such as rice, which is essential to address the nutritional needs of the growing population. It has emerged as the most powerful tool for enhancing crops due to its ability to precisely target and modify specific genes with accuracy, efficiency and simplicity. The key advantage of this technology is that the transgenes responsible for genetic modifications can be easily removed through genetic segregation in one or two generations, ensuring that gene-edited plants are transgene-free like those created through traditional breeding methods. The development of advanced versions of the CRISPR systems like CRISPR-Cpf1, base editing and prime editing shows greater potential for editing rice genomes with even higher precision and efficiency (113). Additionally, the development of CRISPR/*Cas9*-based epigenome editing systems is pushing the boundaries of gene editing to new levels. However, there are still challenges to overcome in applying genome editing to crops. Addressing these challenges will help facilitating the effective use of this technology in crop improvement.

The first challenge in CRISPR-based genome editing is overcoming the strict PAM requirements that limit target sequences. Though the development of alternative PAM sequences and *Cas9* variants, such as x*Cas9*, Sa*Cas9* and Sp*Cas9*-NG, has broadened the scope of genome editing, further development is needed to improve their effectiveness in plants, particularly in rice (114). The next major challenge is the efficient delivery of genetic material, particularly in monocots like rice, where transformation methods like biolistic bombardment and *Agrobacterium*-mediated transformation are hindered by genotype-specific limitations and technical difficulties. Some cultivars are non-responsive to tissue culture and lack regeneration capacity, which demands tissue-culture free methods. While viral vectors and nanomaterials, such as carbon nanotubes and nanoparticles, show promise for improving delivery without tissue culture, further advancements are needed to overcome the existing challenges (115).

Off-target activity is another major concern in CRISPR/*Cas9* gene editing, which might affect the phenotype of interest. Although sequence analysis reveals that off-target mutations in plants are rare, with harmful mutations being eliminated during breeding and beneficial ones retained, it is crucial to implement strategies that minimize off-target effects to maintain specificity. These strategies include designing highly specific sgRNAs, using high-fidelity *Cas9* enzymes like eSp*Cas9*, Sp Cas-HF and employing ribonucleoprotein (RNP) delivery to reduce DNA exposure to CRISPR reagents (116).

The main challenge for the success of genome-edited rice and other crops is reaching the farmers' fields and their performance in natural environments, as most genome editing studies have been confined to controlled settings. Additionally, regulatory uncertainty surrounding gene-edited crops, especially with differing international frameworks, limits adoption. While countries like the USA and some South American nations exempt certain CRISPR-edited crops from GMO regulations, the EU and few countries maintain stringent regulations, hindering progress. A unified global regulatory system is needed to facilitate the widespread use of genome-edited crops (117). Nonetheless, CRISPR/*Cas9* technology holds great promise for improving rice and meeting future global demands. Additionally, consequent research in this field is required to increase the precision of CRISPR which demands collaboration among scientists, policymakers and stakeholders to ensure the responsible and ethical exploration of this technology.

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Authors' contributions

The conceptualization and design of the review was done by JM, KE and KK. JM gathered the literature and drafted the manuscript. Critical revision and supervision were done by UD and MS. KE carried out final verification. All authors read and approved the final manuscript.

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